Novel Arrangement of Enhancer Sequences for NifA-Dependent Activation of the Hydrogenase Gene Promoter in *Rhizobium leguminosarum* bv. viciae

Marta Martinez, Maria-Victoria Colombo, Jose-Manuel Palacios, Juan Imperial, and Tomas Ruiz-Argiieso

Departamento de Biotecnologia and Centro de Biotecnologia y Genomica de Plantas (CBGP), E. T. S. Ingenieros Agronomos, Universidad Politecnica de Madrid, Ciudad Universitaria, sin, 28040 Madrid, Spain, and Consejo Superior de Investigaciones Cientificas, Madrid, Spain

The transcriptional activation of the NifA-dependent cr^{54} promoter of the *Rhizobium leguminosarum* hydrogenase structural genes *hupSL* (P_t) has been studied through gel retardation analysis and detailed mutagenesis. Gel retardation analysis indicated the existence of a physical interaction between NifA and the promoter. Extensive mutagenesis followed by in vivo expression analysis showed that three sequences of 4 bases each (-170 ACAA -167, -161 ACAA -158, and -145 TTGT -142) are required for maximal stimulation of in vivo transcription of the P_1 promoter. The arrangement of these upstream activating sequences (ACAA N_5 ACAA N_{12} TTGT) differs from the canonical 5'ACA N_{10} TGT 3' UAS structure involved in NifA-dependent activation of *niflfix* genes. Mutant promoter analysis indicated that the relative contribution of each of these sequences to P_t promoter activity increases with its proximity to the transcription start site. Analysis of double mutants altered in two out of the three enhancer sequences suggests that each of these sequences functions in NifA-dependent activation of the P_1 promoter in an independent but cooperative mode. The similarities and differences between *cis* elements of *hup* and *niflfix* promoters suggest that the structure of the P_1 promoter has adapted to activation by NifA in order to coexpress hydrogenase and nitrogenase activities in legume nodules.

Gene expression in bacteria is controlled mainly at the transcriptional level, and these organisms have developed a wide variety of promoter sequences to ensure the right products are made in the correct amounts at the appropriate times. Different sigma factors confer promoter specificity and enable the RNA polymerase (RNAP) to distinguish between groups of promoters. Some genes are expressed only under certain conditions. A paramount example is the expression of nitrogen fixation genes in diazotrophic bacteria, which is induced only under the restrictive conditions required for nitrogenase activity. These genes are controlled by a^{S4}-dependent promoters (reviewed in references 5 and 39). Transcription initiation from a^{S4}-type promoters is a multistep process that involves binding of the alternative sigma factor a^{54} (RpoN) to specific promoter sequences at positions —24 (GG)/—12 (TGC) and to the core of the RNAP to form a closed, inactive complex. Transition from a closed to an open RNAP-DNA promoter complex precedes mRNA synthesis and requires the hydrolysis of ATP by an enhancer binding protein (EBP) or promoter activator. The EBPs bind to upstream activator sequences (UAS), enhancer sequences that are located distant (—80 to -150 bp) from the transcriptional start site. EBPs are usually dimeric in their inactive state and need to form higher-grade oligomers for ATP hydrolysis (25). UAS binding was shown to promote higher-grade oligomer formation in some EBPs and to increase the ATP hydrolysis rate (16, 38).

NifA is a member of the EBP family needed for expression of nitrogen fixation *(nif)* and other genes (9, 23). In most cases, NifA binds to a UAS that, in the cognate *nif* genes of nitrogen-fixing bacteria, is a conserved characteristic palindromic transcriptional enhancer, namely, 5' TGT N_{9_u} ACA 3' (1, 6, 21). The UAS-bound NifA is brought into contact with the a^{54}-holoenzyme by DNA looping. This bending event is mediated by the integration host factor (IHF), which binds to a site between the UAS and the core promoter region (15, 30). The NifA protein has a modular structure typical of EBP activators. The N-terminal domain of NifA contains a GAF domain that is common to several cyclic GMP receptors and may be involved in regulation (14). The central domain of NifA shows extensive homology to equivalent domains in other a^{54} activators (22) and belongs to the AAA$^+$ superfamily of ATPases associated with various activity functions. The structure and organization of the AAA$^+$ domain has recently been reviewed (32). The C-terminal domain of NifA is required for enhancer-dependent transcriptional activation, and it contains the helix-turn-helix motif, which is presumed to recognize the UAS (26).

NifA also regulates the expression of the hydrogenase structural genes *hupSL* of *Rhizobium leguminosarum* (3). Uptake hydrogenases are synthesized by some legume-endosymbiotic bacteria (rhizobia) and allow them to recycle the hydrogen generated by nitrogenase (27, 31). The *R. leguminosarum* bv. viciae strain UPM791 contains a cluster of 18 genes, *hupSL*-

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
R. leguminosarum		
UPM791	128C53 Str"	17
E. coli		
BL21(DE3)/pLysS	F *ompT hsdS*$_B$ (r_B m_B) *gal dcm* (DE3) pLysS; Cm"	Invitrogen
ET8000	*rbs lacZ::lSl gyrA hutC*$_K$	18
XL1 Blue	*supE44 hdsRU recAl gyrA46 thi relAl lac F'\proAB*$^+$ *lacP- lacZAM15 TnlO* (Tef)]	12
S17.1	*thi pro hsdR hsdM*$^+$ *recA* RP4 2-Tc::Mu-Km::Tn7	**33**
Plasmids		
pCR2.1-TOPO	PCR product cloning vector; Ap^r, Km"	Invitrogen
pCRnifA	UPM791 *nifA* gene in pCR2.1-TOPO; Ap^r, Km'	19
pMB737/28b$^+$	Expression plasmid of an N-terminal hexahistidine tagged *A. vinelandii* NifA protein; Km"	M. Buck"
pMALcRI	Vector for expression of recombinant fusion proteins in frame to MBP; Ap"	N. E. Biolabs
pMALAnifA	pMALcRI derivative with 222 amino acids of *R. leguminosarum* NifA C-terminal end	Th is work
pMP220	IncP Mob$^+$ Tra~ LacZ; Tc"	34
pHL315	*hupSLdacZ* in pMP220; Tc'	3
pSK315	*hupSL* in pBluescript(SK); Ap"	Th is work
pSKM, pSKD, and pSKT series	*hupSL* with point mutations in pBluescript(SK); Ap"	Th is work
pHLM, pHLD, and pHLT series	*hupSLwlacZ* with point mutations in pMP220; Tc"	Th is work
TOPO-SPE series	P_1 promoter deletions in pCR2.1-TOPO; Ap^r, Km"	Th is work
pSPV4	pMP220 derivative; Tc^r	24
pSPE series	P_1 promoter deletions fused to *lacZ* in pSPV4; Tc"	Th is work
pMJ220	*K. pneumoniae* NifA in pACYC184; Cm'	21

[a] Imperial College of Science, Technology and Medicine, London SW7 2BB, United Kingdom.

CDEFGHIJK hypABFCDEX, that are required for hydrogenase synthesis. The hydrogen oxidation capacity is efficiently induced in symbiosis with peas, but not in free-living cells, and the regulation of this expression has been studied in detail . The hydrogenase structural genes *hupSL* are temporally and spatially coexpressed with the nitrogenase structural genes in pea root nodules, suggesting the existence of common inducing signals and activating factors (4). Regulation studies have shown that the symbiosis-specific transcription of *hupSL* genes occurs from a NifA-dependent, —24/—12 (σ^{-54})-type promoter (P_x) located upstream of *hupS* (3, 13). These studies allowed the identification of a DNA region (positions —173/-88 relative to the transcription start site) essential for Y_1 activity. However, no canonical UAS (5' TGT N_{10} ACA 3') were found in or around this regulatory region. In this paper, we report that three upstream 4-base sequences, spaced in a noncanonical mode (-170 ACAA N_5 ACAA N_{12} TTGT -142) are involved in activation of P_x by NifA in an independent but cooperative way.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *R. leguminosarum* strains were routinely grown in tryptone-yeast extract or yeast extract-mannitol medium at 28°C. *Escherichia coli* strains were grown in Luria-Bertani medium. Antibiotics were added at the following final concentrations (fig ml^{-1}): tetracycline, 12 (6 for *Rhizobium);* kanamycin, 50; chloramphenicol, 20; and ampicillin, 100. *Klebsiella pneumoniae* NifA was aerobically expressed from plasmid pMJ220 *inE. coli* strain ET8000 at 28°C as previously described by Morett and Buck

DNA manipulation and analysis. Plasmid DNA preparation, restriction and enzyme digestions, transformation of DNA into *E. coli* cells, DNA cloning, PCR DNA amplification, and agarose and polyacrylamide gel electrophoresis were performed by standard procedures . Plasmid transfer from *E. coli* into *Rhizobium* strains was carried out by conjugation using *E. coli* S17.1 as the donor strain. DNA sequencing was carried out by using a Rhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI377 automatic sequencer (PE Biosystems, Foster City, CA) or by using a Sequenase kit (Sequenase, version 2.0: United States Biochemicals, Cleveland, OH).

Generation of P_1 promoter deletions and mutants. Serial deletions of the Pj promoter were generated by nested-PCR amplification of the promoter region, using DNA from plasmid pHL315 as a template, the upper primers listed in Table 2, and the lower primer SPER (5'-CTTGCTCCTCCAGCAATCCC-3'). The PCR products were cloned as EcoRI fragments in the reporter vector pSPV4, following an intermediate cloning step in the pCR2.1-TOPO vector, thus generating the pSPE plasmid series (Fig. 2).

To generate site-directed mutants, a 2.4-kb KpnI-SphI fragment from pHL315, containing the *hupSL* promoter region, the *hupS* gene, and part of *hupL,* was subcloned as a Hindlll fragment into plasmid pBluescript SK, resulting in plasmid pSK315. A set of derivative plasmids containing mutant promoters (the pSKM series, pSKDl, and pSKD2) were generated by replacing in the wild-type promoter region each of the single-nucleotide bases from positions -172 to -140 or 2 bases simultaneously (-169/-160 and -168/-159) with their complementary bases using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. In each case, synthetic oligonucleotides (listed in Table SI in the supplemental material) and their respective complementary oligonucleotides were used to synthesize the entire plasmid by using a pSK315 construct as a template. Plasmids containing pSKD3 double and pSKT3 triple mutations were generated by using oligonucleotides corresponding to the —143 single mutation and pSKM4 and pSKDl constructs, respectively, as templates. The —172 to —140 promoter regions of the resulting mutant plasmids were sequenced to confirm the presence of the expected mutations.

Recombinant fusion protein construction. The pMALAnifA plasmid was generated by cloning in pMALcRI, in frame with the MalE coding region, a 670-bp EcoRI-BamHI DNA fragment obtained from the pCRnifA plasmid. This fragment contains the coding sequence for the 222-amino-acid C-terminal region of NifA.

DNA binding assays. A 300-bp EcoRI DNA fragment from plasmid pSPEl, containing the *hupS* upstream region (the —243 to +57 region), was labeled with

TABLE 2. Upper primers used in the P_1 promoter deletions

PCR product length (bp)	Upper primer		
	Designation	Sequence	5′ position[a]
300	SPE1	5′-CTTCCGCGAGCCTTGGTGAC-3′	−243
241	SPE2	5′-CGGAATTCAGGCAAGGCGATGACAAAGT-3′	−184
224	SPE3	5′-AAGTCTACAAGTGACGTCGC-3′	−167
215	SPE4	5′-AGTGACGTCGCCATTGTCTG-3′	−158
197	SPE5	5′-TGCTTGATGCGCCGAGTTCA-3′	−140
181	SPE6	5′-TTCAATCCCGCTTATCCGGT-3′	−124
165	SPE7	5′-CGGTTCTCTTGCCGCCGCCA-3′	−108
145	SPE8	5′-TCCTCAAGGTAAACATTAAA-3′	−88

[a] Position relative to the *hupS* transcription start site.

[a-^{32}P]dATP using the Klenow fragment of DNA polymerase I and used as a probe in gel retardation assays. In these assays, we used an N-terminal hexahistidine-tagged *Azobacter vinelandii* NifA protein obtained through expression of the pMB737/28b+ plasmid *inE. coli* cells and further metal affinity purification performed as previously described . Alternatively, we used crude cell extracts from *E. coli* cultures expressing a fusion between the maltose-binding protein (MBP) and the 222-amino-acid peptide of the C-terminal region of *R. leguminosamm* UPM791 NifA.

DNA binding reactions were performed in a final volume of 20 u,l by mixing the DNA probe (3 nM) with the above-mentioned NifA derivatives in a buffer containing 100 mM potassium glutamate, 10 mM magnesium acetate, 50 ug ml^{-1} bovine serum albumin, 0.5 M 3-mercaptoethanol, 5% glycerol, 50 mM EDTA, 50 ^g ml^{-1} herring sperm DNA, 25 mM Tris-acetate, pH 7.6. The reaction mixture was incubated at 37°C for 15 min. DNA-protein complexes were separated from free DNA by electrophoresis in a 6% polyacrylamide-10% glycerol gel run in Tris-borate-EDTA buffer at 200 V and 4°C for 45 min.

RESULTS

Binding of NifA to the P_1 promoter DNA region. Previous Pj promoter deletion experiments demonstrated that the DNA region located between positions —173 and —88 relative to the *hupS* transcription initiation site was essential for V_1 activation . With the aim of demonstrating directly the binding of NifA to this region, a 300-bp DNA fragment containing nucleotides from position —243 to +57 was used in band shift experiments. In these assays, we first used purified N-terminally hexahistidine-tagged *A. vinelandii* NifA protein. The presence of this protein in the binding reaction was associated with a retarded band (Fig. 1A). A second set of experiments were carried out with a homologous NifA DNA binding domain using *E. coli* cell extracts containing a fusion protein between the MBP and a 222-amino acid peptide from the C-terminal end of NifA from *R. leguminosamm* bv. viciae UPM791 (MBP-CNifA). In these experiments, a specific band with different mobility was detected, and its intensity correlated with the amount of extract present in the reaction mixture (Fig. IB). No band was detected when the extract was obtained from *E. coli* cells lacking the MBP-CNifA fusion protein. These results indicate that NifA interacts with the V_1 promoter, likely inducing *hupSL* expression directly and not by modulating the synthesis of an intermediate activator.

Deletion analysis of the promoter region. To better define the region essential for NifA-dependent activation of the Pj promoter, a detailed deletion analysis of the —183/—88 DNA region was carried out (Fig. 2). The (3-galactosidase activity associated with nested deletions of the V_1 promoter was determined in aerobically grown *E. coli* cells expressing the *K. pneumoniae* NifA, and also in pea bacteroids from *R. leguminosamm* UPM791 (Fig. 2). In the absence of NifA, the expression associated with pSPEl was reduced to background levels. Deletion of DNA fragments containing sequences upstream of position —183 (fusions pSPEl and pSPE2) had no effect on Pj activity. Further deletions led to significant reductions in Pj activity. Fusions pSPE3, pSPE4, and pSPE5 exhibited 20, 51, and 83% reductions of the wild-type (3-galactosidase activity, respectively. A similar pattern of expression of the pSPE fusions containing the P_x promoter deletions was obtained in *R. leguminosamm* pea bacteroids (Fig. 2). These results, along with those obtained previously , indicate that the region required for NifA-dependent activation of the V_1 promoter spans positions —172 to —140.

Identification of sequences required for NifA-dependent transcriptional activation by site-directed mutagenesis. In order to identify the specific bases involved in NifA-dependent activation of $P_{1;}$ we used *lacZ* fusion constructions harboring a set of promoter derivatives with single point mutations in each of the DNA bases of the —172 to —140 promoter region (plasmids pHLMl to pHLM33 in Fig. 3). In all cases, the

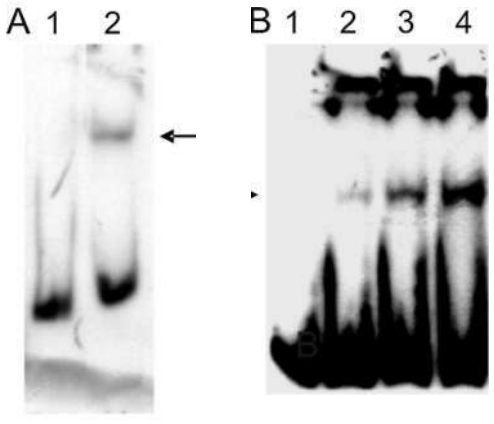

FIG 1. Binding of NifA to the *hupSL* promoter. Shown are the results of gel retardation assays carried out with a DNA fragment of 300 bp containing the *hupS* upstream region from positions —243 to +57. The DNA probe was incubated with purified A *vinelandii* six-His NifA (37) (lanes: 1, control with no NifA; 2, 200 ng of six-His NifA) (A) or crude *E. coli* cell extracts containing a fusion between the MBP and a 222-amino-acid peptide from the C-terminal end of NifA of *R. leguminosamm* bv. viciae strain UPM791 (lanes: 1, control with cell extract without MBP-CNifA; 2, 3, and 4, probe incubated with 50,125, and 250 ng of protein, respectively) (B). The migration positions of the potential DNA-NifA complex are indicated by arrows.

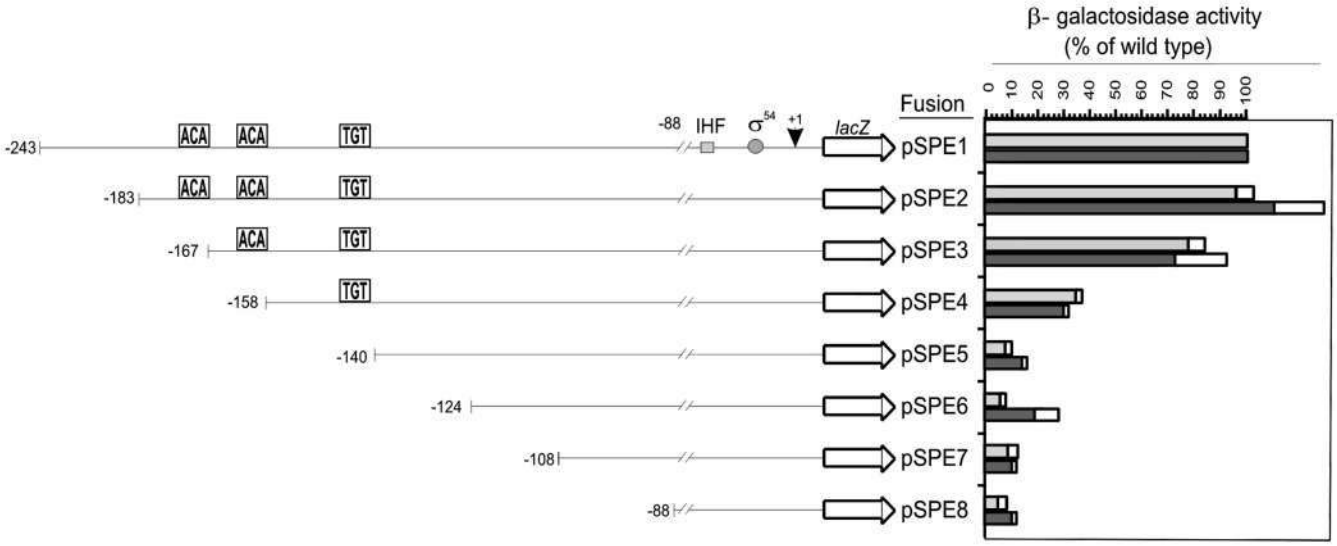

FIG. 2. Deletion analysis of the P_x promoter. Deletion variants present in the different *hupS-lacZ* fusion constructs (pSPE plasmids) are shown on the left. The locations of the IHF- and cr^{54}-binding sequences are highlighted, and the transcriptional start site (+1) is marked by a vertical arrowhead. The ACA and TGT triplets are boxed. The (3-galactosidase activities associated with the different fusions were determined in aerobically grown *E. coli* cultures expressing *K. pneumoniae* NifA (light-gray bars) and in *R. leguminosarum* UPM791 bacteroids prepared from pea nodules (dark-gray bars). The values are presented as percentages of the levels of (3-galactosidase activity associated with the undeleted fusion construct pSPEl (ca. 1,600 Miller units in *E. coli* cells and ca. 400 Miller units in pea bacteroids). The values are the averages of three replicate experiments, and the white bars represent the standard errors.

mutation replaced the original base with its complementary one. These fusions were tested for p-galactosidase activity in *E. coli* ET8000 expressing *K. pneumoniae* NifA (Fig. 3). In these experiments, no P_x promoter activity was observed in the absence of NifA. Data obtained in these experiments (Fig. 3) revealed that mutations in three sequences of 4 bases each (-170 ACAA -167, -161 ACAA -158, and -145 TTGT -142) were associated with significant decreases in p-galacto-

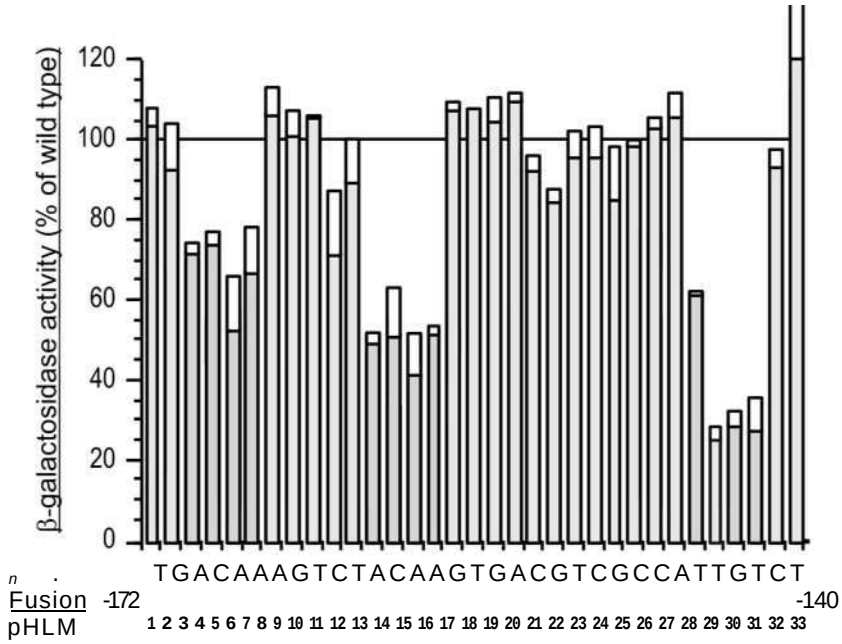

FIG. 3. Point mutational analysis of the P_x promoter. Promoter mutants were generated by replacing each of the nucleotide bases of the -172 to -140 wild-type promoter region with its complementary base. The mutant promoters carrying single base substitutions were fused to the *lacZ* gene, and the resulting plasmids (pHLM series) were tested for (3-galactosidase activity in *E. coli* cells expressing *K. pneumoniae* NifA. In the diagram, the nucleotide modified in each case is shown in the abscissa, and the gray bar above each nucleotide indicates the corresponding relative level of (3-galactosidase activity, expressed as a percentage of that of the wild-type promoter. The values represent the averages of three replicate determinations, and the standard errors are represented by empty bars. The nucleotide bases corresponding to the HSSs required for NifA-dependent activation of P_x are shown in boldface letters.

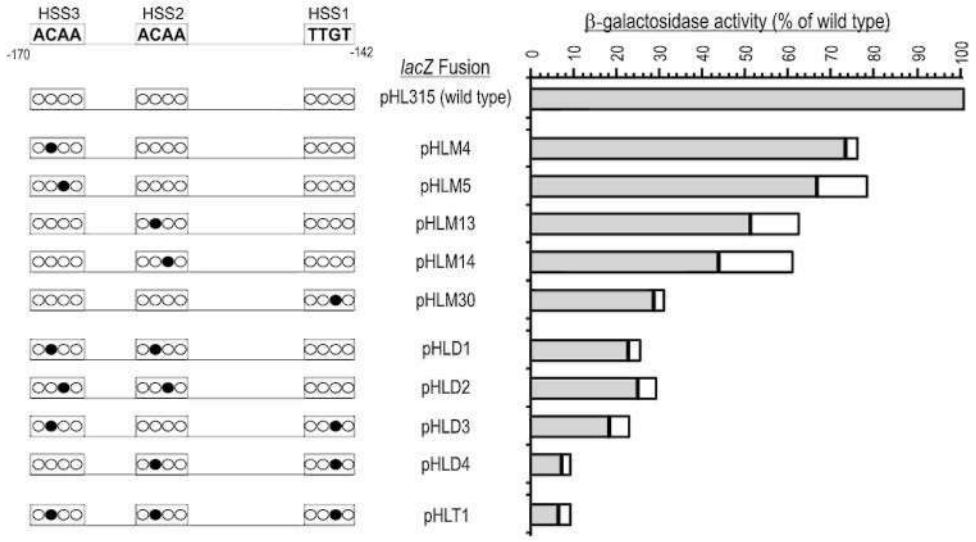

FIG. 4. Analysis of interactions among HSSs for NifA-dependent V_1 promoter expression. Relevant regions of the *hupSL-lacZ* fusions containing mutations in the HSSs are represented on the left. The relative location and the bases of each of the three identified HSSs in the Pj promoter are shown on top. The mutated DNA fragments, generated by replacing a single base in one, two, or three of the HSSs with their complementary base(s), are shown below. The mutated bases are shown as boldface circles and the wild-type bases as white circles. The horizontal bars on the right represent relative fj-galactosidase activities associated with each pHL plasmid, expressed as percentages of that of the wild-type promoter, when tested in *E. coli* cells expressing *K. pneumoniae* NifA. The shaded bars depict the average values of fj-galactosidase activities, and the white bars indicate the standard errors.

sidase activity, indicating that these sequences were involved in the regulation of the NifA-dependent P_x activity. These sequences are similar to the half-sites described in the canonical NifA-binding UAS. Fusions containing single mutations in -145 TTGT (half-site sequence 1 [HSS1]), -161 ACAA (HSS2), and -170 ACAA (HSS3) exhibited ca. 30, 50, and 70%, respectively, of the activity associated with the wild-type promoter, which is consistent with data from deletion analysis of the promoter (Fig. 2). The relative (3-galactosidase activities associated with mutant promoters also indicated that the contribution of each HSS to V_1 activity increased with its proximity to the transcription start site. These sequences are likely the only ones essential for NifA-dependent activity of the V_1 promoter, since an extensive random mutagenesis of a DNA fragment extending from positions —183 to +57 failed to identify positions involved in this process other than IHF- and a^{54}-binding regions

Since the three identified HSSs were involved in NifA-dependent activation of the V_1 promoter, it was of interest to investigate whether they had an independent or cooperative contribution to promoter activation. With this aim, we analyzed different combinations of mutations affecting more than one HSS (Fig. 4). Fusions containing two mutated HSSs (plasmids of the pHLD series) showed significantly reduced Pj activity compared to the corresponding mutations in only one HSS. Furthermore, the effect of altering HSS1 was very significant in the presence of wild-type HSS2 and HSS3 (70% reduction with pHLM30), indicating that HSS1 is the most relevant site for activation. However, the presence of an intact HSS1, when combined with altered HSS2 and HSS3, resulted in low Pj activation (ca. 25% with pHLDl and pHLD2), suggesting that HSSl-dependent PI activation is cooperatively stimulated by HSS2/HSS3.

Finally, and in order to ascertain the role of each HSS in the original genetic background, a selection of single-, double-, and triple-mutant promoter fusions were introduced into *R. leguminosarum* strain UPM791 and tested for (3-galactosidase activity in pea bacteroids. In this background, we observed a pattern of (3-galactosidase activity similar to that described in *E. coli* (data not shown).

DISCUSSION

In this paper, we have shown the existence of a physical interaction between NifA and the *hupSL* promoter region of *R. leguminosarum.* These results, together with previous data , demonstrate a direct regulation of the hydrogenase structural genes *hupSL* by NifA, expanding the range of genes whose expression is directly activated by this protein (9, 23, 35).

Detailed deletion and mutagenesis experiments have led to the identification of the sequences required for NifA-dependent transcription of the *hupSL* genes (—170 ACAA N_5 ACAA N_{12} TTGT —142). These sequences are similar to the half-sites present in canonical NifA UAS (5' TGT N_{10} ACA 3') found in most nitrogen-fixing bacteria , including *R. leguminosarum* bv. viciae strain UPM791 . Considering the distance between the NifA-binding half-sites, the distribution 5' ACA N_6 ACA N_{14} TGT 3' found in P_x differs from the canonical arrangement of NifA-binding sequences of *nif* and *fix* gene promoters. The transcription analysis using double mutants affected in more than one HSS showed independent but cooperative effects of the different HSSs on V_1 promoter activation. Based on these results, we postulate that NifA binds independently to each of the HSSs, with a higher affinity for the HSS closer to the transcription start site. Since NifA binding to DNA occurs through the TGT motif , different NifA

protein units are expected to bind to such sequences located in the top and bottom DNA chains in the *hupSL* promoter.

The requirement for an oligomerization process to attain the active form of transcriptional activators of the NtrC/NifA family has been demonstrated i. According to our data, it can be postulated that NifA forms an oligomer that binds with different affinities to each of the three HSSs in the PI promoter. The stability of such oligomer-DNA complexes would determine the observed effects of the different mutations on V_1 promoter activity, with this activity being maximal in the presence of the three intact HSSs. Although the assays with single mutant promoters clearly established the independent participation of the three HSSs in the activation of V_1 *by K. pneumoniae* NifA, the existence of more than one NifA oligomer is unlikely because of space limitations due to the proximity of the different half-sites in the DNA.

The arrangement of HSSs in the V_1 promoter is not restricted to *R. leguminosarum* strain UPM791, since this DNA region is conserved in several *R. leguminosarum* strains from different origins . Also, it has to be remarked that the Pj structure is efficiently recognized by NifA proteins from different bacteria, as a similar pattern of transcription of V_1 variants was observed in *E. coli* cells (harboring *K. pneumoniae* NifA) and in *R. leguminosarum* bacteroids (harboring their own NifA). These results also suggest that the differences between NifA C-terminal domains from these two species are not relevant for the recognition of the HSSs.

The meaning of the differences between P_x and *nifH* promoter structures is not evident. It may be related to the need to achieve different rates of expression of hydrogenase and nitrogenase genes to ensure adequate concentrations of each enzyme. It has been previously demonstrated that canonical NifA-controlled promoters, such as *nifH,* exhibited significantly higher activities than V_1 in expression analyses carried out in *E. coli* cells . This could be a consequence of a higher stability of the DNA-NifA oligomer complex in the *nifH* promoter due to the distance between the TGT and ACA sequences, which might facilitate the binding of NifA. Differences in the structures of *hup* and *nif* promoters might also reflect the different origins of the systems. It has been proposed that *hup* genes are the result of a process of horizontal gene transfer from other bacteria . This conclusion was based on the presence in *R. leguminosarum* strain UPM791 of a *hoxA* pseudogene. HoxA is involved in H_2-dependent signal transduction for hydrogenase expression under free-living conditions in other bacteria i. The incoming gene cluster might have adapted to NifA activation in order to coordinate H_2 oxidation with symbiotic H_2 production by nitrogenase, thus losing its original regulation system, from which only *hoxA* traces remain. This adaptation process has likely led to a sophisticated promoter, such as $P_{1;}$ that allows simultaneous hydrogenase and nitrogenase expression regulated by NifA in pea nodules. This prediction is consistent with the "regulatory-noise" hypothesis , which proposes that transcriptional control systems develop responsiveness to new signals by a process that involves not only the regulators, but also the DNA sequences.

ACKNOWLEDGMENTS

This work has been funded by research projects from Spain's Ministers de Education y Ciencia (BIO2004-05385 to J.I. and BIO2004-00251 to J.-M.P.) and from Comunidad de Madrid (S-505/AMB/0321 MICROAMBIENTE-CM) to T.R.-A.

We are grateful to M. Buck for the six-His-NifA expression system.

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